Final report

Grant/Contract Title: (YIP-08) ACCELERATING THE RATE-LIMITING STEP IN NOVEL ENZYMATIC CARBOHYDRATE-TO-HYDROGEN TECHNOLOGY BY ENZYME ENGINEERING

Grant/Contract Number: FA9550-08-1-0145

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Technical Report

1. Background

One of the greatest challenges of the sustainability revolution is the production of sustainable affordable transportation fuels from renewable resource. Lignocellulosic biomass is the most abundant (less costly collectable) bioresource. The annual chemical energy stored in phytobiomass is approximately 30 times of annual transportation energy consumption. The cost-effective utilization of a small fraction of biomass could achieve transportation fuel independence based on local renewable resource and provide a nearly carbon-neutral solution.

Cell-free synthetic pathway biotransformation (SyPaB) is the implementation of complicated biochemical reactions by *in vitro* assembly of enzyme and coenzymes. Different from *in vivo* synthetic biology, SyPaB has numerous advantages, such as high product yield, fast reaction rate, great engineering flexibility, and so on, but it suffers from a lack of thermostable enzymes and costly labile coenzymes. The SyPaB can implement some biochemical reactions that microbes cannot do, such as high-yield production of hydrogen, electricity, and polyols.

2. Key discovery/accomplishment/result/findings

1. **Demonstration of the highest-yield hydrogen production from cellulosic materials and water by using in vitro synthetic biology platform and a 10-fold increase in enzymatic hydrogen generation rate**

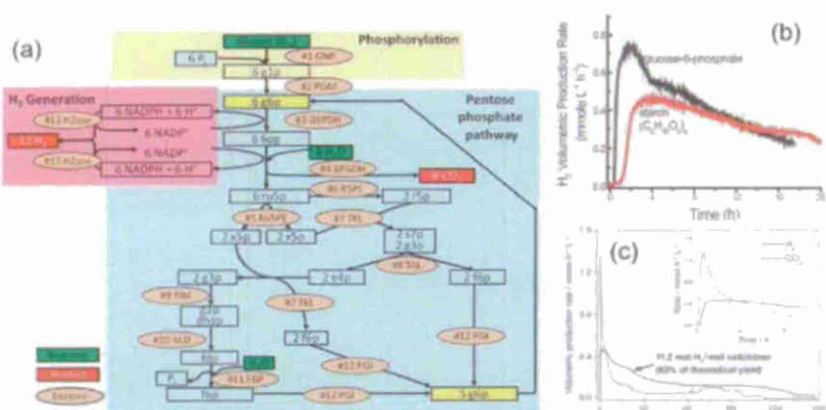


Figure 1. The cell-free synthetic enzymatic pathway (a), high-yield generation of hydrogen from starch (b) (*1*) or soluble cellodextrin (c) (*2*).

2. **Validation of numerous enzymes with enough stability for low-cost hydrogen production**

For example, one-step purification and immobilization of a thermostable phosphoglucose isomerase has a very long time with a total turn-over number of 10^9 mol of product/mol of enzyme (*3*).

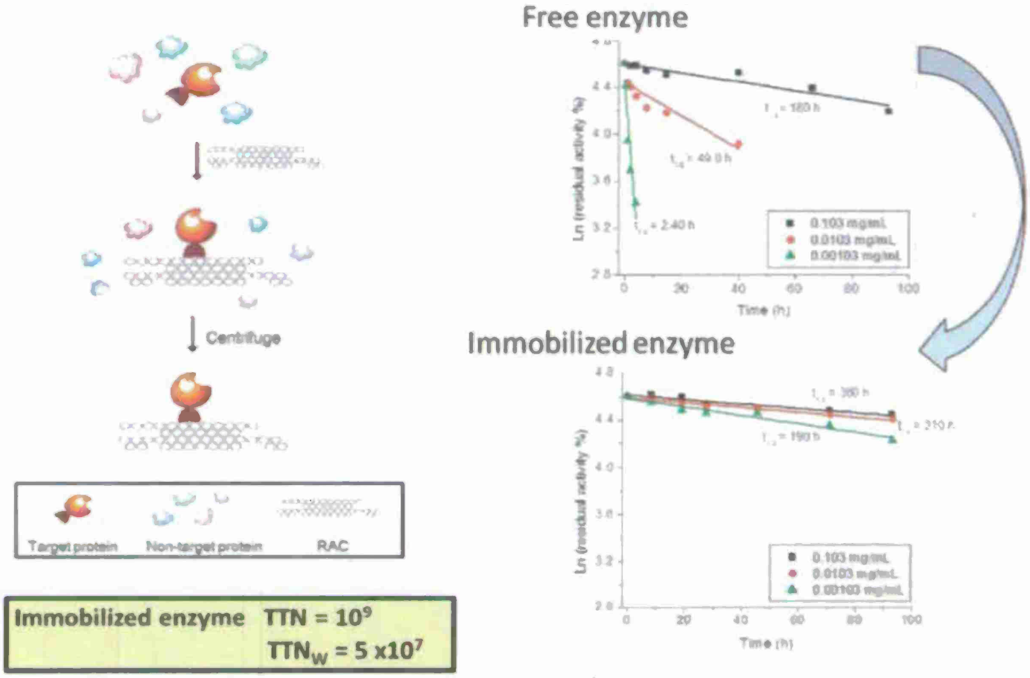
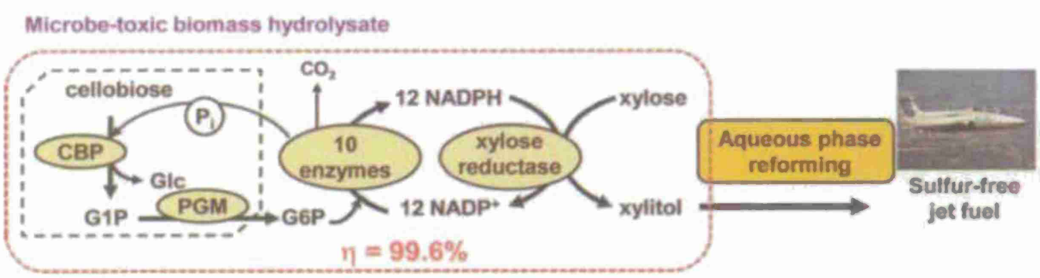


Figure 2. One-step protein purification and immobilization.

3. **Development of the most energy efficient way for the production of low-surfur jet fuel from biomass sugars by a hybrid of biocatalysis and catalysis.**

A new hybrid of biocatalysis and catalysis has been demonstrated for the production of jet fuel from soluble biomass sugars – dilute acid pretreated microbe-toxic biomass hydrolysate containing xylose, cellobiose, acetate, furfural, phenolic compounds biomass sugars. This new pathway has a combined efficiency of as high as 94%, much higher than any other biofuels (fatty acid ethyl esters or bioalkanes) produced by microbes (*4*). A 13-enzyme cocktail was able to convert xylose to xylitol by using cellobiose as a hydrogen source in the presence of microbe-toxic impurities (*5*).



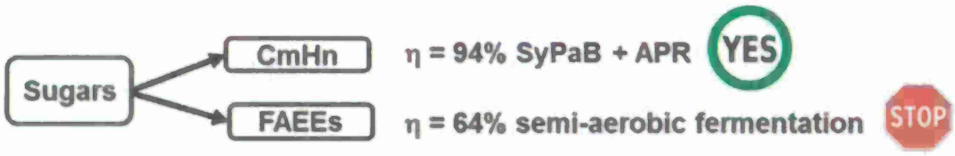


Figure 3. A new high-energy retaining approach to produce jet fuel by a hybrid of biocatalysis and catalysis (A) and the theoretical yield comparison of jet fuel production by this new way with microbial biodiesel developed by Jay Keasling (B) (*4, 6*).

4. Conduct energy efficiency analysis and predict the role of biomass.

In conclusion, a small fraction of the USA biomass resource would be sufficient to replace all imported crude oil if we can increase biomass utilization efficiency greatly as shown below (sugar fuel cell vehicle) (*7*).

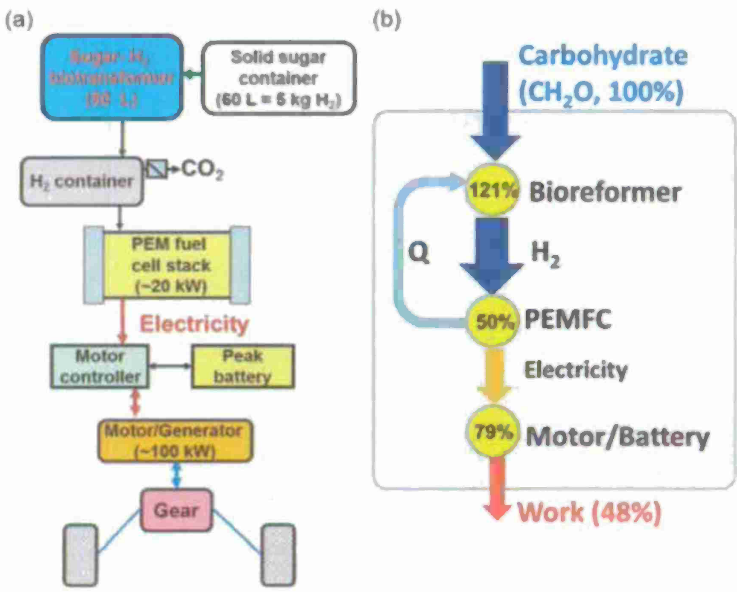


Figure 4. The hypothetical sugar fuel cell vehicle (A) and energy efficiency analysis of the power train system (B).

5. Development of high-energy density biodegradable enzymatic fuel cells (EFCs).

EFCs have received increasing interest as a next-generation environmentally friendly power source. The highest power densities (e.g., by Sony Co.) have reached ~5-10 mW/cm^2 of anode, sufficient for powering many portable electronics. However, current EFCs only partially oxidize the complicated six-carbon glucose molecule using one or two enzymes (i.e. 2-4 moles of electrons produced per mole glucose), resulting in low energy density of sugar batteries. If complete oxidation of sugar is achieved (e.g. 24 moles of electrons per mole of glucose), the energy density of EFCs can reach as high as 380 Wh/kg for a 20% (w/v) sugar/water slurry, approximately three times of those of lithium ion batteries (~130 Wh/kg). High energy density sugar batteries would compete with primary batteries, secondary batteries, and direct methanol fuel cells (DMFC). Bioinspired sugar batteries featuring complete oxidation of sugar may revolutionize the power source, especially for portable electronics. In the future, miniaturized fully-developed sugar batteries may replace most secondary batteries and DMFC. Refilling sugar slurry in a sugar battery is much faster and safer than recharging secondary batteries. Compared to DMFC, sugar slurry is not toxic and its energy density is higher than a ~1 M methanol solution. Sugar batteries would be nearly 100% biodegradable except electrode and wire and would not require any special (exported or costly) metals, such as lithium or rare earth metals.

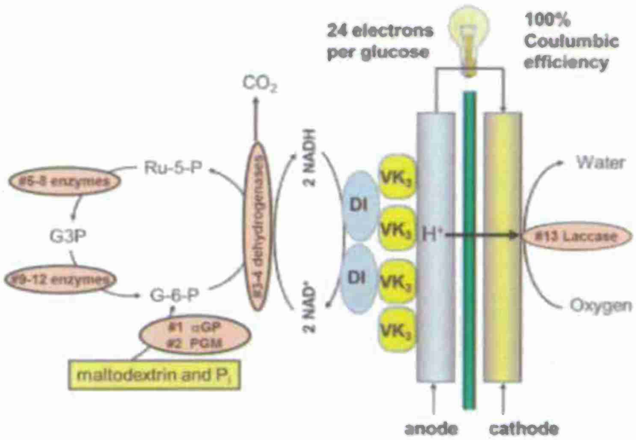


Figure 5. Scheme of enzymatic fuel cell featuring a patent-pending synthetic enzymatic pathway that can completely oxidize starch to CO2 and generate 24 electrons per glucose.

3. Collaborators (cell-free system)

Shelley Minteer, St. Louis University
Plamen Atanassov, UNW
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Jian-Jiang Zhong, Shanghai JiaoTong University, China
Yan Feng, Shanghai JiaoTong University, China
Jibin Sun, Tianjin Institute of Industrial Biotechnology, China

4. Honors/awards

PI Awards

2011	Virginia Tech's College of Engineering Faculty Fellow Award
2010	Daniel I.C. Wang Award (Biotechnology and Bioengineering and ACS BIOT)
2009	Sunkist Engineering Designer Award (ASABE)
2008	British Petroleum Young Scientists Award (IBS 2008)
2008	DuPont Young Faculty Award
2008	Outstanding new faculty award (College of Engineering of Virginia Tech)

Student honor

2011	Winner, Climate Leadership Challenge 2011 (Patrick Kirk, David Osmalov, Joseph Keuler, and Matthew Kirk – UW Madison Team, $50,000 cash award)

5. Tech transfers

A biofuel start-up company Gate Fuels Inc. is negotiating with VTIP office about the license of sugar battery. Gate Fuels is submitting the NSF SBIR Phase I proposal.

Shell GameChange Program decides to fund the PHASE I Sweet Hydrogen ($450,000, 1 year). Also, it is negotitating the sugary hydrogen license from VTIP.

6. Publications mainly sponsored by AFOSR

(1) Zhang Y-HP*. 2011. Chapter 8: Hydrogen production from carbohydrates: a mini-review. ACS Symposium Series 1067:203-216. (Sustainable Production of Fuels, Chemicals, and Fibers from Forest Biomass), Oxford University Press, UK.

(2) Zhang Y-HP*. 2011. What is vital (and not vital) to advance economically-competitive biofuels production. *Process Biochemistry* 46: 2091-2110 (Invited opinion review).

(3) Liao HH, Myung S, Zhang Y-HP*. 2011. One-step purification and immobilization of thermophilic polyphosphate glucokinase from *Thermobifida fusca* YX: glucose-6-phosphate generation without ATP. *Applied Microbiology and Biotechnology* Epub, DOI: 10.1007/s00253-011-3458-1

(4) Zhang Y-HP*, Myung S, You C, Zhu ZG, Rollin J. 2011. Toward low-cost biomanufacturing through cell-free synthetic biology: bottom-up design. *Journal of Materials Chemistry* Epub. DOI:10.1039/C1JM12078F (Invited feature article).

(5) Zhang Y-HP*. 2011. Substrate channeling and enzyme complexes for biotechnological applications. *Biotechnology Advances* 29: 715-725.

(6) Ye X, Zhu ZG, Zhang CM, Zhang Y-HP*. 2011. Family 9 carbohydrate-binding module improves the catalytic potential of *Clostridium thermocellum* cellodextrin phosphorylase on cellulosic materials. *Applied Microbiology and Biotechnology* 92:551-560.

(7) Zhang Y-HP*. 2011. Simpler is better: high-yield and potential low-cost biofuels production through cell-free synthetic pathway biotransformation (SyPaB). *ACS Catalysis* 1: 998-1009 (Invited perspective).

(8) Huang WD, Zhang Y-HP*. 2011. Energy efficiency analysis: biomass-to-wheel efficiency related with biofuels production, fuel distribution, and powertrain systems. *PLoS One* 6(7): e22113 (Analysis).

(9) Zhu ZG, Wang YR, Minteer SD, Zhang Y-HP. 2011. Maltodextrin-powered enzymatic fuel cell through a non-natural enzymatic pathway. *Journal of Power Sources* 196:7505-7509.

(10) Myung S, Zhang X-Z, Zhang Y-HP*. 2011. Ultra-stable phosphoglucose isomerase through immobilization of cellulose-binding module-tagged thermophilic enzyme on low-cost high-capacity cellulosic adsorbent. *Biotechnology Progress* 27:969–975.

(11) Wang YR, Huang WD, Sathisuksanoh N, Zhu ZG, Zhang Y-HP*. 2011. Biohydrogenation from biomass sugar mediated by cell-free synthetic pathway biotransformation. *Chemistry and Biology* 18: 372-380 (Featured article).

(12) Huang WD, Zhang Y-HP*. 2011. Analysis of biofuels production from sugar based on three criteria: Thermodynamics, bioenergetics, and product separation. *Energy & Environmental Science*. 4:784-792. (Analysis)

(13) Zhang Y-HP*, Mielenz JR. 2011. Renewable hydrogen carrier – carbohydrate: constructing the carbon-neutral carbohydrate economy. *Energies* 4:254-275 (Invited Perspective).

(14) Zhang Y-HP*. 2010. Renewable carbohydrates are a potential high density hydrogen carrier. *International Journal of Hydrogen Energy* 35:10334-10342.

(15) Zhang Y-HP*, Sun J-B, Zhong J-J. 2010. Biofuel production by *in vitro* synthetic enzymatic pathway biotransformation. *Current Opinion in Biotechnology* 23: 663-669. (Invited Review)

(16) Myung S, Wang YR, Zhang Y-HP*. 2010. Fructose-1,6-bisphosphatase from a hyper-thermophilic bacterium *Thermotoga maritima*: Cloning, characterization, metabolite stability, and its implications. *Process Biochemistry* 45:1882-1887.

(17) Ye X, Zhang Y-HP*. 2010. Thermophilic α-glucan phosphorylase from *Clostridium thermocellum*: Cloning, characterization, and enhanced thermostability. *Journal of Molecular Catalysis B: Enzymatic* 65:110–116.

(18) Zhang Y-HP.* 2010. Production of biocommodities and bioelectricity by cell-free synthetic enzymatic pathway biotransformations: Challenges and opportunities. *Biotechnology and Bioengineering* 105:663-677.

(19) Wang Y*, Rollin JA, Zhang Y-HP. 2010. Enhancing allele-specific PRC for specific detection of DNA mutants with nucleotide deletion and insertion. *Molecular and Cellular Probes* 24:15-19.

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(23) Zhang Y-HP*. 2009. A sweet out-of-the-box solution to the hydrogen economy: Is sugar-powered car science fiction? *Energy and Environmental Science* 2: 272-282. (Invited perspective)

(24) Ye X, Wang Y, Hopkins RC, Adams MWW, Evans BR, Mielenz JR, Zhang Y-HP*. 2009. Spontaneous high-yield production of hydrogen from cellulosic materials and water catalyzed by enzyme cocktails. *ChemSusChem* 2: 149-152.

(25) Hong J, Ye X, Wang Y, Zhang Y-HP*. 2008. Bioseparation of recombinant cellulose binding module-protein by affinity adsorption on an ultra-high-capacity cellulosic adsorbent. *Analytica Chimica Acta* 621:193-199.

(26) Hong J, Wang Y, Ye X, Zhang Y-HP*. 2008. Simple protein purification through affinity adsorption on regenerated amorphous cellulose followed by intein self-cleavage. *Journal of Chromatography A*. 1194(2): 150-154.

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1. Y.-H. P. Zhang, B. R. Evans, J. R. Mielenz, R. C. Hopkins, M. W. W. Adams, *PLoS One* **2**, e456 (2007).
2. X. Ye *et al.*, *ChemSusChem* **2**, 149 (2009).
3. S. Myung, X.-Z. Zhang, Y.-H. P. Zhang, *Biotechnol. Prog.* **27**, 969 (2011).
4. Y.-H. P. Zhang, *Proc. Biochem.* **46**, 2091 (2011).
5. Y. Wang, W. Huang, N. Sathitsuksanoh, Z. Zhu, Y.-H. P. Zhang, *Chem. Biol.* **18**, 372 (2011).
6. W. D. Huang, Y.-H. P. Zhang, *Energy Environ. Sci.* **4**, 784 (2011).
7. W. D. Huang, Y.-H. P. Zhang, *PLoS One* **6**, e22113 (2011).

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14. ABSTRACT
Carbohydrate, which is renewable, carbon-neutral, and evenly distributed, will replace oil because of lower costs ($/GJ), better performance in the transport sector, better safety, and more applications (e.g., hydrogen carrier and electricity storage compound). Via AFOSR support, several goals were accomplished (1) demonstration of the highest-yield hydrogen production from cellulosic materials and water by using in vitro synthetic biology platform, (2) a 10-fold increase in enzymatic hydrogen generation rate, (3) validation of numerous enzymes with enough stability for low-cost hydrogen production (i.e., total turn-over number of 10E7-10E9 mol of product/mol of enzyme), and (4) development of the most energy efficient way for the production of low-sulfur jet fuel from biomass sugars by a hybrid of biocatalysis and catalysis. Furthermore, the energy efficiency suggested that a small fraction of the USA biomass resource (i.e., 7%) would be sufficient to replace all imported crude oil if biomass utilization efficiency was increased greatly.

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